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1 **Genetic monitoring of pilot whales, *Globicephala spp.* (Cetacea:**
2 **Delphinidae), stranded on French coasts**

3 **Etienne Sabatier⁽¹⁾, Eric Pante⁽¹⁾, Claire Dussud⁽²⁾, Olivier Van Canneyt⁽²⁾, Benoit**
4 **Simon-Bouhet⁽¹⁾, and Amélia Viricel^{(1)*}**

5 **(1)** Littoral, Environnement et Sociétés, UMR 7266, CNRS-Université de La
6 Rochelle, 2 rue Olympe de Gouges, 17000 La Rochelle, France

7 **(2)** Observatoire PELAGIS, UMS 3462, CNRS-Université de La Rochelle, Pôle
8 analytique, 5 allée de l'océan, 17000 La Rochelle, France

9 *Corresponding author: Amélia Viricel. Email address: amelia.viricel@gmail.com

10 **Keywords:** forensics; genetic monitoring; marine mammal; mitochondrial DNA;
11 stranding

12 **Abstract:** We used mitochondrial DNA control region sequences to genetically identify
13 to the species level 23 pilot whales (genus *Globicephala*) that stranded on French coasts
14 between 1996 and 2011. Genetic analysis complemented morphological diagnoses,
15 often hampered by an overlap in morphological characters between the two species or
16 incomplete measurements. Mitochondrial DNA data allowed identification of 21 long-
17 finned pilot whales (*Globicephala melas*) and two unusual stranding events of the more
18 tropical species (*Globicephala macrorhynchus*). In pilot whales as in most cetaceans,
19 shifts in species range are expected to occur due to global climate change. In this
20 context, our study contributes to the long-term monitoring of pilot whale stranding
21 events, providing indirect information on species occurrence.

22 Every year, several hundreds to one thousand marine mammals strand on French
23 coasts (Van Canneyt et al. 2012). Each stranding is recorded by the Joint Service Unit
24 PELAGIS (La Rochelle, France). The French marine mammal stranding network
25 (coordinated by PELAGIS) performs species identifications and necropsies of stranded
26 animals in an effort to monitor the marine mammal biodiversity and to report evidence
27 of potential anthropogenic impacts (e.g. marks from incidental catch from fisheries). In
28 cases of badly decomposed carcasses, or whenever there is ambiguity in morphological
29 identification, genetic data can aid in the species identification process (Alfonsi et al. In
30 Press). In this short communication, we complemented morphological species diagnoses
31 with genetic data to assist with the monitoring of pilot whale (genus *Globicephala*,
32 Lesson 1828) stranding events.

33 The genus *Globicephala* is comprised of two species that have opposite
34 distribution patterns: the long-finned pilot whale (LFPW: *Globicephala melas*, (Traill
35 1809)) and the short-finned pilot whale (SFPW: *Globicephala macrorhynchus*, Gray
36 1846) (Wilson and Reeder 2005). The LFPW has an anti-tropical distribution and is
37 present in cold temperate waters of the Atlantic Ocean, the Mediterranean Sea and the
38 southern hemisphere (Fullard et al. 2000), while the SFPW inhabits tropical, and warm
39 temperate waters all around the globe (Kasuya and March 1984). The two species co-
40 occur in a region of overlap in the North Atlantic Ocean, and the Bay of Biscay
41 represents the northern limit of the SFPW's distribution range (Nores and Pérez 1988;
42 Gonzalez et al. 2000). Furthermore, two extant subspecies of LFPW are recognized: *G.*
43 *melas melas* (Traill 1809) (North Atlantic LFPW) and *G. melas edwardii*, Smith 1834
44 (Southern LFPW) (Wilson and Reeder 2005). Stranding events of LFPW on French
45 coasts are a common phenomenon with 10 to 30 cases reported every year.

46 Comparatively, stranding events of SFPW are rare (Van Canneyt et al. 2012). Indeed,
47 the first stranding event of a SFPW in France was reported in 1966 (Duguy 1968),
48 followed by another one in 1988 (Van Canneyt et al. 2012). More recently, two
49 stranding events have been reported on the French Atlantic coast: one in 2008, and one
50 (pending species confirmation) in 2011 (Van Canneyt et al. 2012).

51 In the current context of global climate change, shifts in distribution ranges are
52 expected to occur for most cetacean species (MacLeod 2009). As pilot whales'
53 distribution is correlated with water temperature (e.g. Hoydal and Lastein 1993), their
54 species range may indeed be indirectly affected by ongoing changes in sea-surface
55 temperature. In this context, long-term monitoring of pilot whale stranding events can
56 be used as a proxy to detect potential shifts in their distribution, especially to follow the
57 evolution of the northern limit of the SFPW in the Atlantic Ocean.

58 Morphological features used to identify pilot whales to the species level are the
59 number of teeth per half jaw (LFPW: 9-12; SFPW: 7-9), and the ratio of the length of
60 the pectoral fin to the total length of the body (LFPW: 18-27%; SFPW: 14-19%) (Van
61 Bree 1971; Robineau 2005). However, the range overlap in these morphological
62 features hampers species diagnosis. Thus, genetic analysis appears to be a good
63 complementary tool to the morphological approach. We re-examined species
64 identification of past pilot whale stranding events using genetics to complement
65 morphological diagnoses and confirm some of the recent unusual stranding events of
66 SFPW that occurred on French coasts.

67 The mitochondrial DNA (mtDNA) control region can be used to reliably
68 distinguish between the two species of pilot whales thanks to six diagnostic
69 substitutions (Oremus et al. 2009). Thus, we sequenced a portion of the mtDNA control

70 region to genetically identify the species of 23 pilot whales that stranded on French
71 coasts between 1996 and 2011 and for which tissue samples had been collected (Table
72 1: voucher information provided by PELAGIS). DNA extractions were performed from
73 approximately 25 mg of skin, muscle or kidney tissue preserved in ethanol using a
74 Nucleospin Tissue kit (Macherey-Nagel EURL, Hoerdt, France) following the
75 manufacturer's protocol. PCR amplifications were performed using primers L15824
76 (5'-CCTCACTCCTCCCTAAGACT-3') (Rosel et al. 1999) and H16498 (5'-
77 CCTGAAGTAAGAACCAGATG-3') (Rosel et al. 1994), and the PCR profile
78 described in Vollmer et al. (2011). The 50 μ L reactions included 50 ng genomic DNA,
79 0.3 μ M for each primer, 0.15 mM dNTPs (Euromedex, Mundolsheim, France), 10 mM
80 Tris-HCl (pH 8.3), 50 mM KCl, 0.1% Triton X-100, 1.5 mM MgCl₂, and 2 U Taq
81 polymerase (VWR, Fontenay sous Bois, France). PCR products were sent to
82 Genoscreen (Lilles, France) for purification and Sanger sequencing (in both directions).
83 Sequences were edited and aligned by eye using the BioEdit Sequence Alignment
84 Editor v. 7.1.11 (Hall 1999). Mitochondrial DNA control region sequences from
85 Oremus et al. (2009) published on Genbank were used as a reference dataset to identify
86 the species and haplotype of each sample. Thus, the final sequence alignment was
87 reduced to 345 base-pairs (bp) to match the portion analyzed in Oremus et al. (2009).

88 Among these 23 individuals, morphological information was either not available
89 or incomplete for 18 individuals. For the remaining specimens, the species could not be
90 determined using morphology for three individuals, as their measurements lay in the
91 range of overlap between the two species, or the ratio and number of teeth used in the
92 key were in contradiction yielding ambiguity in species diagnosis (Table 1). For the
93 latter specimen however, we considered measurements as unreliable due to the

94 advanced state of decomposition of the body (Table 1), which prevented accurate
95 morphometric analysis. A map of stranding locations for the 23 specimens was
96 constructed using the marmap package v. 0.5 (Pante and Simon-Bouhet 2013) in R v.
97 3.0.1 (R Development Core Team 2013).

98 A complete haplotype of the mtDNA control region (345 bp) was successfully obtained
99 for all individuals. The 23 individuals were characterized by 4 different haplotypes: 2
100 LFPWs haplotypes referenced by Oremus et al. (2009) as haplotypes R (GenBank:
101 FJ513345) and S (GenBank: FJ513346), and 2 SFPWs haplotypes referenced as
102 haplotypes A (GenBank: FJ513328) and D (GenBank: FJ513331). Species
103 identification made using genetic data matched morphological identification (when
104 applicable) suggesting that the morphological key is reliable when measurements are
105 not within the range of overlap between the two species. Sequences allowed us to
106 genetically identify or to confirm the species of all individuals, resulting in 21 LFPWs
107 and 2 SFPWs (Table 1 and Figure 1). Thus, genetic analysis appears to be a
108 complementary tool to the morphological key, and this highlights the importance of
109 collecting tissue samples after every stranding event to allow genetic analysis whenever
110 morphological identification is ambiguous or impossible. It is important to note,
111 however, that unless nuclear loci are analyzed, one cannot rule out hybrid ancestry for
112 individuals presenting morphological characters in the zone of overlap or of ambiguous
113 phenotype, as illustrated by a recent study reporting an interspecific hybrid between the
114 two *Globicephala* species (Miralles et al. 2013).

115 To place our sequences in a worldwide phylogeographic context, we compared
116 mtDNA control region haplotype frequencies observed in this study, with those
117 obtained by Oremus et al. (2009). For LFPWs we sequenced 2 different haplotypes, R

118 (n=3) and S (n=18), while Oremus et al. (2009) reported 3 haplotypes for LFPWs in the
119 North Atlantic Ocean: S (n=56), X (n=3) and P (n=11). Thus, haplotype S was the most
120 common haplotype for North Atlantic LFPWs in both studies. Interestingly however,
121 haplotype R had only been reported in LFPWs from the southern hemisphere (i.e., *G.*
122 *melas edwardii*). Thus, we report an additional shared haplotype between the two *G.*
123 *melas* subspecies compared to Oremus et al. (2009), and our study elevates to four the
124 number of haplotypes found in LFPWs from the North Atlantic. The two haplotypes
125 sequenced in our study for SFPWs (haplotype A: n=1; haplotype D: n=1) had been
126 previously reported in Oremus et al. (2009) for individuals sampled in the North
127 Atlantic. However, haplotype A had been mostly sequenced in individuals from the
128 Pacific Ocean (n=21) and was found for a single individual from the Atlantic Ocean
129 (Oremus et al. 2009). Comparatively, a recent study conducted on SFPWs biopsy-
130 sampled from 14 groups around Madeira reported that all 29 individuals sequenced
131 shared haplotype A (Alves et al. 2013).

132 In conclusion, our study constitutes a first step toward genetic monitoring of the
133 two species of pilot whales along French coasts, a region of particular importance as it
134 constitutes the northern limit of the SFPW's species range.

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203

204 **Table 1:** Voucher information for 23 pilot whales genetically analysed in this study. For
 205 each individual, the voucher number, stranding date and location (latitude and
 206 longitude), and body state of decomposition are shown (State: 1: stranded alive and
 207 died within 48 hours; 2: freshly dead; 3: dead and decomposed; 4: dead and highly
 208 decomposed; NA: not available.). Morphological data (M.) were categorized as:
 209 available and unambiguous (A), incomplete (I), available but characters were in the
 210 zone of overlap (O), ambiguous due to characters in contradiction (*), not available
 211 (NA). The last columns report the species identification made using morphology
 212 (M. ID) when possible, the genetic species identification (G. ID), and the
 213 individual's mtDNA control region haplotype (Hap: names according to Oremus et
 214 al. 2009). LFPW: *Globicephala melas*; SFPW: *Globicephala macrorhynchus*.

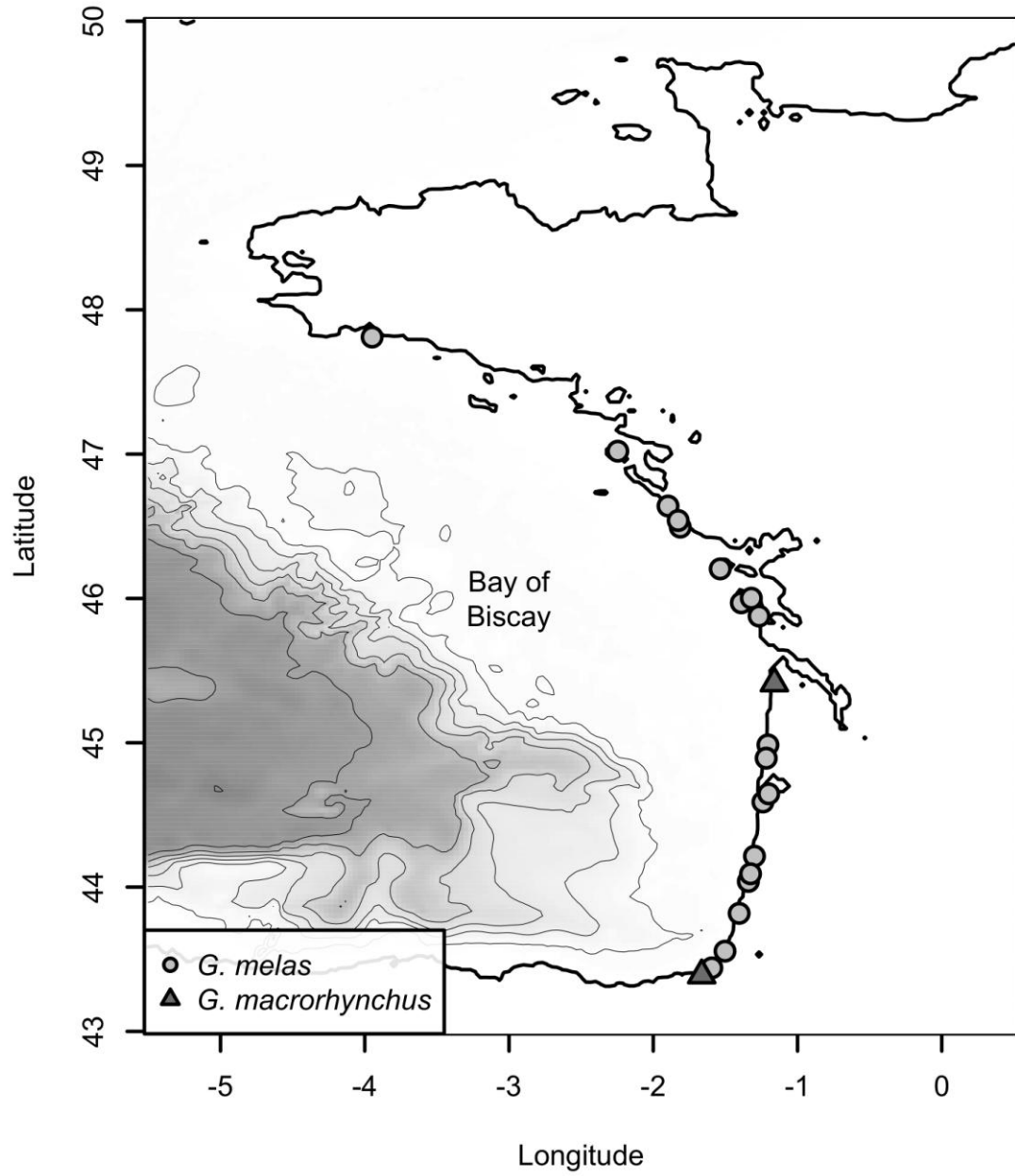
Voucher	Date	Lat.	Long.	State	M.	M. ID	G. ID	Hap
9601009	30-Jan-96	44.9855	-1.2052	2	A	LFPW	LFPW	S
9706105	22-Jun-97	45.9696	-1.3899	2	NA		LFPW	S
9904034	19-Apr-99	45.8777	-1.2666	3	NA		LFPW	S
9912065	17-Dec-99	46.2049	-1.5359	2	NA		LFPW	S
10104070	13-Apr-01	46.6406	-1.8977	2	NA		LFPW	S
10206217	20-Jun-02	44.5894	-1.2398	3	I		LFPW	S
10404055	21-Apr-04	44.8933	-1.2171	2	I		LFPW	S
10505023	01-May-05	46.4940	-1.8127	NA	NA		LFPW	S
10512100	28-Feb-05	43.8181	-1.4047	NA	O		LFPW	S
10602023	21-Feb-06	44.2144	-1.2987	3	I		LFPW	S
10706033	27-Jun-07	46.5400	-1.8273	3	NA		LFPW	S
10712119	13-Mar-07	44.0395	-1.3407	4	*		LFPW	R
10803050	16-Mar-08	44.6463	-1.1999	2	O		LFPW	S
10804078	25-Apr-08	44.8933	-1.2171	2	I		LFPW	R
10809120	16-Sep-08	43.3895	-1.6643	1	A	SFPW	SFPW	A
10902053	11-Feb-09	44.0395	-1.3407	3	I		LFPW	R
10903073	11-Mar-09	43.5562	-1.5028	1	I		LFPW	S
10903078	11-Mar-09	44.0921	-1.3257	1	I		LFPW	S
10907116	09-Jul-09	47.8111	-3.9489	3	NA		LFPW	S
11007041	19-Jul-10	46.0020	-1.3203	2	NA		LFPW	S
11108074	02-Aug-11	45.4126	-1.1604	1	I		SFPW	D
11111087	24-Jun-11	43.4405	-1.5939	2	NA		LFPW	S
11112093	21-Dec-11	47.0204	-2.2461	1	NA		LFPW	S

215 **Figure legend**

216 **Figure 1:** Stranding locations of 23 pilot whales (*Globicephala* spp.) genetically
217 identified using mtDNA control region sequences.

218

219 **Figure 1.**



220